

Alanine Aminotransferase Homologs Catalyze the Glutamate:Glyoxylate Aminotransferase Reaction in Peroxisomes of Arabidopsis¹

Aaron H. Liepman² and Laura J. Olsen*

Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109-1048

Plant peroxisomal glyoxylate aminotransferases play central roles within the photorespiratory pathway. Genes encoding glyoxylate aminotransferases have been isolated from several animals and microbes, but only recently have plant homologs been identified. Three Arabidopsis homologs of alanine (Ala):glyoxylate aminotransferase 2 (AGT2) contain a putative type 1 peroxisomal targeting signal (PTS1), but the metabolic significance of these AGT2 homologs is unknown. GGT1 and GGT2 are Ala aminotransferase (AlaAT) homologs from Arabidopsis that represent another type of glyoxylate aminotransferase. These proteins are class I aminotransferases, each containing a putative PTS1. GGT1 and GGT2 are members of a small family of AlaATs in Arabidopsis. When expressed as recombinant proteins in *Escherichia coli*, GGT1 and GGT2 displayed biochemical characteristics very similar to one another, and to the Arabidopsis protein purified from leaves. Four aminotransferase activities were specifically associated with GGT1 and GGT2, using the substrate pairs glutamate (Glu):glyoxylate, Ala:glyoxylate, Glu:pyruvate, and Ala:2-oxoglutarate. GGT1 and GGT2 may have partially redundant functions; transcripts of both genes were detected in many of the same tissues. Although Glu:glyoxylate aminotransferase (GGT) activity has been observed in several locations in different plants and algae, including the cytoplasm and mitochondria, our subcellular fractionation data indicate that GGT activity was exclusively peroxisomal in Arabidopsis. Thus, glyoxylate aminotransferase reactions in plant peroxisomes appear to be catalyzed by at least two distinct types of aminotransferases: an AGT1 homolog with serine:glyoxylate aminotransferase activity (A.H. Liepman, L.J. Olsen [2001] Plant J 25: 487–498), and a pair of closely related, potentially redundant AlaAT homologs with GGT activity.

Photorespiration is a metabolite salvage pathway coordinated among at least three organelles in plant cells: chloroplasts, peroxisomes, and mitochondria. In the process of recycling phosphoglycolate, generated by the oxygenase activity of Rubisco, into the photosynthetically useful metabolite phosphoglycerate, this pathway may consume a significant proportion of the energy generated by photosynthesis in C₃ plants (Ogren, 1984; Husic et al., 1987; Buchanan et al., 2000). Despite the obvious importance of photorespiration, genes encoding several enzymes of this pathway have not been identified.

Leaf peroxisomes are hypothesized to contain at least four aminotransferase activities including Ser:glyoxylate aminotransferase (SGT), Glu:glyoxylate aminotransferase (GGT), Ala:glyoxylate aminotransferase (AGT), and Asp:2-oxoglutarate aminotransferase (AspAT; Rehfield and Tolbert, 1972). Studies in which isolated plant peroxisomes were fed labeled

glycolate and various amino acids, in conjunction with studies of photorespiration mutants, have demonstrated that peroxisomal glyoxylate aminotransferases occupy central roles in photorespiration (Kisaki and Tolbert, 1969; Somerville and Ogren, 1980; Chang and Huang, 1981; Betsche, 1983; Liang and Huang, 1983; Yu et al., 1984).

Although genes encoding glyoxylate aminotransferases have been isolated in various animals and microbes (Nishiyama et al., 1990; Chistoserdova and Lidstrom, 1994; Lee et al., 1995; Hagashita et al., 1996), genes encoding plant homologs had not been characterized until recently (Liepman and Olsen, 2001). Animals contain two structurally distinct types of AGTs: AGT1 and AGT2. AGT1 is a class IV aminotransferase that is localized to peroxisomes, mitochondria, or both organelles, depending upon dietary habit (Danpure, 1997), whereas AGT2 is a class II aminotransferase localized exclusively to mitochondria in animals (Takada and Noguchi, 1980; Noguchi and Mori, 1981; Ogawa et al., 1990; Lee et al., 1995). We demonstrated previously that an AGT1 homolog with SGT activity is located in peroxisomes, where it is involved in the photorespiratory pathway in Arabidopsis. Kinetic analysis of Arabidopsis AGT1 suggests that this protein mainly uses the substrates Ser and glyoxylate. Although AGT1 catalyzes an AGT reaction in vitro, its high K_m value for Ala

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² Present address: Department of Energy Plant Research Lab, Michigan State University, East Lansing, MI 48824-1312.

* Corresponding author; e-mail ljo@umich.edu; fax 734-647-0884.

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indicates that Ala may not be a physiological substrate for this enzyme (Liepman and Olsen, 2001).

Recent studies of Arabidopsis glyoxylate aminotransferases were preceded by a number of studies in which these enzymes were purified and characterized from a variety of plants (Rehfield and Tolbert, 1972; Noguchi and Hayashi, 1981; Nakamura and Tolbert, 1983; Paszkowski and Niedzielska, 1989; Roberts and Lees, 1997). There has been some confusion regarding the identities of these aminotransferases because of varying degrees of enzyme purity and the ability of these enzymes to catalyze reactions with multiple amino donor:acceptor combinations. Previous reports suggest that enzymes possessing GGT and AGT activities also catalyze Glu:pyruvate aminotransferase (GPT) and Ala:2-oxoglutarate aminotransferase (AKT) reactions (Noguchi and Hayashi, 1981; Biekmann and Feierabend, 1982; Paszkowski and Niedzielska, 1989; Orzechowski et al., 1999). GPT and AKT activities are typically attributed to Ala aminotransferases (AlaATs; EC 2.6.1.2). Much of the previous research on plant AlaATs has been characterization of chromatographically purified preparations from many different plant and algal sources. These studies indicate that all AlaATs possess AKT and GPT activities, and that a subset of the AlaATs also catalyzes GGT and AGT reactions.

Plants contain multiple AlaAT isoforms, localized to various subcellular locations including peroxisomes, mitochondria, and the cytosol. Between two and six forms of AlaAT have been observed in plant tissues (Gazeu-Reyjal and Crouzet, 1976; Noguchi and Hayashi, 1981; Biekmann and Feierabend, 1982; Penther, 1991; Son et al., 1991; Otter et al., 1992; Watson et al., 1992; Orzechowski et al., 1999). In general, it appears that AlaATs with glyoxylate aminotransferase activity are localized to peroxisomes, whereas those lacking glyoxylate aminotransferase activity are extraperoxisomal (see also Biekmann and Feierabend, 1982; Noguchi and Fujiwara, 1982; Yokota et al., 1985).

The presence of several AlaAT isozymes localized within different subcellular compartments in plants suggests that these enzymes may play roles in a number of metabolic pathways. Some isozymes appear to be expressed constitutively and play roles in general amino acid metabolism (Penther, 1991; Otter et al., 1992). There is also evidence that other isozymes may have more specialized roles. Several groups have observed light-dependent expression of plant AlaAT isozymes, indicating potential involvement in photorespiration (Noguchi and Fujiwara, 1982; Penther, 1991; Son et al., 1991; Otter et al., 1992; Son and Sugiyama, 1992). A cytosolic AlaAT isozyme in *Panicum miliaceum*, with increased expression during greening, is found in both mesophyll and bundle sheath cells, and has been implicated in the transfer of C₃ units between these cell types (Son et al., 1991; Son and Sugiyama, 1992). An AlaAT lacking glyoxy-

late aminotransferase activity is induced by hypoxia in barley (*Hordeum vulgare*), corn (*Zea mays*), and *P. miliaceum* roots (Good and Muench, 1992; Muench and Good, 1994; Muench et al., 1998). Elevated levels of Ala are observed in oxygen-deficient roots (Hoffman et al., 1986); Ala production may be an effective mechanism used by plants to conserve nitrogen and carbon atoms that would be lost by ethanol glycolysis (Good and Muench, 1992; Muench et al., 1998). In rice (*Oryza sativa*), an AlaAT gene is expressed in the starchy endosperm tissue of maturing seeds, where it may serve a key role in amino acid conversion for seed storage protein production (Kikuchi et al., 1999).

We have previously identified AGT1, the peroxisomal glyoxylate aminotransferase that catalyzes the SGT reaction of photorespiration (Liepman and Olsen, 2001). At least one additional distinct glyoxylate aminotransferase within the photorespiratory pathway has been implicated in biochemical and genetic studies (Somerville and Ogren, 1980; Nakamura and Tolbert, 1983; Yu et al., 1984). The objectives of this study were to identify the gene(s) encoding Arabidopsis GGT and to determine the subcellular localization and substrate specificity of this enzyme. Here, we report the identification of a pair of Arabidopsis genes encoding closely related GGTs belonging to a small group of AlaATs in Arabidopsis. We also describe kinetic and transcript expression analyses of these multispecific aminotransferases and the subcellular distribution of GGT activity in Arabidopsis. Although genes encoding AlaATs have been isolated from a number of plants and other organisms (Son and Sugiyama, 1992; Muench and Good, 1994; Muench et al., 1998; Kikuchi et al., 1999), this is the first report, to our knowledge, describing the identification of genes encoding a GGT from any organism.

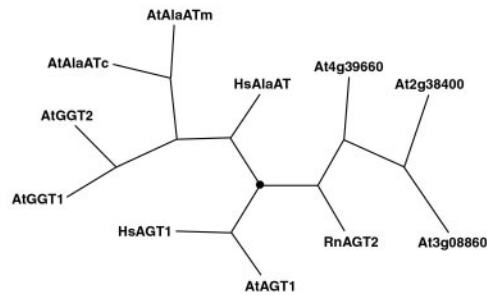
RESULTS

Arabidopsis AGT2 Homologs

Although GGT activity is well documented in plants, the identity of the enzyme that catalyzes this reaction is unknown. It was demonstrated previously that purified enzyme preparations from various plants that contained GGT activity also often contained AGT activity. In animals, there are two structurally distinct aminotransferases that catalyze the AGT reaction, AGT1 and AGT2 (Noguchi et al., 1978). Because the Arabidopsis AGT1 homolog did not catalyze the AGT reaction with physiologically relevant kinetics (Liepman and Olsen, 2001) and did not display any GGT activity (A.H. Liepman and L.J. Olsen, unpublished data), a logical candidate for a plant AGT/GGT could be an AGT2 homolog. Three Arabidopsis AGT2 homologs (At2g38400, At3g08860, and At4g39660) were identified using rat (*Rattus norvegicus*) AGT2 to query the databases (Fig. 1).

All three Arabidopsis AGT2 homologs are class II aminotransferases, and have predicted molecular

A.



B.

At4g39660	-----MALQR	QLLKRAISDI	YHRRRAISLLR	TDFSTSPSIA	DAPPHIPPVF	HQPRPYKGPS	ADEVLQKRKK	FLGPSLFHY	QKPLNIVEGK	85
At2g38400	---MQRFAAK	RSVQNISVSL	WRRCISSTSQ	AATASVKQSD	EFOARLPFFA	YTPPPYTGPS	ADVILSKRKE	FLSPSMFCLY	RKPLNIVDGK	87
At3g08860	MRKLTAVNSL	LKRNNYLLPR	HGSSQTAQR	TSSVRETETE	TKLPKMPFFN	YSPPPYDGPS	TAEIIAKRRE	FLSPALFHY	NTPLNIVEAK	90
RnAGT2	MSLAWRTLQK	AFYLETSLRI	LQMRPFLSCA	SRIYVPLKLT	HTKHNMPFCD	FSPEKYQSLA	YNHVLEIHKQ	HLSPVNTAYF	QKPLLLHQGH	90
cons.	90
At4g39660	MQYLDESGR	RYLDAFAGIV	TVSCGHCHPD	ILNAITEQSK	LLOHATTIYL	HHAIGDFAEA	LAAKMPGNLK	VVYFVNSGSE	ANELAMMMAR	175
At2g38400	MQYLFDESGR	RYLDAFAGIA	VVNCGHCHPD	VVEPVINQIK	RLQHPITVLYL	NHAIADFSEA	LASKLPGLDK	VVFTTNSGTE	ANELALMMAK	177
At3g08860	MQYVFDENGR	RYLDAFGGIA	TVSCGHCHPE	VVNSVVKQIK	LINHSTILYL	NHTISDFAEA	LVSTLPGLDK	VVFTTNSGTE	ANELAMMMAR	180
RnAGT2	MEWLPFSEGN	RYLDFPFGIV	TVGVGHCHPK	VTAVAKKQMD	RLWHTSVVFF	HSPMHEYAER	LSALLPEPLK	VIFLVNSGSE	ANDLAMVMMAR	180
cons.	*.....*	************	180
At4g39660	LYTGSLEMIS	LRNAYHGGSS	NTIGLTALNT	WKYPLP-QGE	IHHVVPNDPY	RGVFG-----	-----SDG	SLYAKDVQDH		242
At2g38400	LYTGCQDIVA	VRNGYHGNA	ATMGATQSQM	WKFNVV-QNS	VHHALNPDPY	RGVFG-----	-----SDG	EKYAKDLQDL		244
At3g08860	LYTGCNDIVS	LRNSYHGNA	ATMGATAQSN	WKFNVV-QSG	VHHAINDPDPY	RGIFG-----	-----SDG	EKYASDVHDL		247
RnAGT2	AYSNHDTIIS	FRGAYHGCSP	YTLGLTNVGI	YKMKVPSTIA	CQSTMCPDVF	RGPWGGSHCR	DSPVQTVRKC	SCAPDGCQAK	ERYIEQFKDT	270
cons.	*.....*	************	270
At4g39660	IEYGTSGKVA	GFAIETIQGV	GGAVELAPGY	LKSVEIVNR	AGGVCIADDEV	QTGFGRGTGS	YWGFTQDQV	PDIVTMAKGI	GNGLPLGAVV	332
At2g38400	IQYGTGTHIA	GFICEAIQGV	GGIVELAPGY	LSAAYDVTVK	AGGLFIADDEV	QSGFARTG-N	FWGFEAHNV	PDIVTMAKGI	GNGLPLGAVV	333
At3g08860	IQFGTSGQVA	GFIGESIQQV	GGIVELAPGY	LPAAYDIVRK	AGGVCIADDEV	QSGFARTGTH	FWGFQSHGVI	PDIVTMAKGI	GNGLPLGAVV	337
RnAGT2	LNTSVATSLA	GFFAEPIQGV	NGVVQYKPEF	LKEALALVRE	RGGVCIADDEV	QTGFGRGTGS	YWGFTQDQV	PDIVTMAKGI	GNGLPLGAVV	360
cons.*	************	360
At4g39660	TTPEIASVLA	SKI-LFNTFG	GNPVCSAGGL	AVLNVIDKEK	RQEHCAEVGS	HLIQLRLKDVQ	KRHDIIGDVR	GRGLMVGIEL	VSDRKDKTPA	421
At2g38400	TTPEIAGVLT	RRS-YFNTFG	GNVSSTTAGL	AVLNVIDKEK	LQENAMVGS	YLKEKLTQLK	EKHEIIGDVR	GRGLMLGVEL	VSDRKDKTPA	422
At3g08860	TTPEIAGVLS	RRS-YFNTFG	GNPMCTAAGH	AVLRVLVHEK	LQENANLVGS	HLKRLTLK	NYELIGDVR	GRGLMLGVEL	VSDRKDKTPA	426
RnAGT2	TTPEIASVLA	KHLHFFSTFG	GSPLACAIGS	AVLEVEEEN	LQRNSQEVGT	YMLLKFAKLR	DEFDIVGDVR	KGKLLWGIEM	VQDKISRPPL	450
cons.	******	*****	********	******	450
At4g39660	-KAETSVLFE	QLRELGLLVG	KGGLHGNVFR	IKPFMCFTKD	DADFLVDALD	YSISRL----	----			476
At2g38400	-TAETLHIMD	QMKELGVLLG	KGGYFGNVFR	ITPPLCFTKD	DADFLVEAMD	YSMSKM----	----			477
At3g08860	-KAETLHIMD	QMKEMGVLLG	KGGYFGNVFR	ITPPLCFTLS	DADFLVDVMD	HAMSKM----	----			481
RnAGT2	PKTEVNQIHE	DKDMGLLVG	RGGNFSQTFR	IAPPMRVTKL	EVDLAEVFR	SALTQHMERR	AK			512
cons.*	*****	*****	**********	512

Figure 1. Sequence analysis of Arabidopsis aminotransferases. A, Unrooted tree containing members of the AGT1, AGT2, and AlaAT families was constructed using the ClustalW 1.75 program at the Genebee server (<http://www.genebee.msu.su/clustal/advanced.html>). The origin of the unrooted tree is indicated with a black circle. Default settings were used for tree construction, except that Kimura's correction was enabled. The AGT2 homologs from Arabidopsis are designated by their Arabidopsis Genome Initiative (AGI) locus numbers (At2g38400, At3g08860, and At4g39660). The mammalian homologs closest to each type of Arabidopsis aminotransferase are shown. RnAGT2, rat AGT2 (accession no. Q64565); HsAGT1, human (*Homo sapiens*) AGT1 (accession no. 178273); HsAlaAT, human AlaAT (accession no. P24298); AtAGT1, Arabidopsis AGT1 (AGI locus At2g13360); AtGGT1, Arabidopsis GGT1 (AGI locus At1g23310); AtGGT2, Arabidopsis GGT2 (AGI locus At1g70580); AtAlaATc, cytosolic isozyme of Arabidopsis alanine aminotransferase (AGI locus At1g17290); and AtAlaATm, mitochondrial isozyme of Arabidopsis alanine aminotransferase (At1g72330). B, Arabidopsis AGT2 homologs were aligned with rat AGT2 (RnAGT2) to produce a consensus sequence (cons.) using the ClustalW algorithm. Asterisks denote residues identical in all four sequences; dots indicate residues similar in at least two of the sequences. Black circles have been placed above putative aminotransferase consensus residues; K320 of At4g39660 is the predicted pyridoxal-5'-phosphate (PLP) cofactor-binding site. A solid bar above the carboxyl terminus of the three Arabidopsis AGT2 homologs indicates the putative PTS1.

weights of approximately 52 kD. Based on sequence alignment and comparison with solved structures of other class II aminotransferases, residues G264, D290 or E291, K320, and R450 of Arabidopsis AGT2 homolog At4g39660 correspond to the conserved residues involved in enzymatic catalysis and binding of the cofactor PLP. Pair-wise comparison of these sequences with each other indicates that the Arabidopsis AGT2 homologs are 60% to 71% identical and 89% to 93% similar to one another. The Arabidopsis AGT2 homologs are 37% to 40% identical and 72% to 74%

similar to the rat sequence (Lee et al., 1995). Expressed sequence tags (ESTs) of these genes are much lower in abundance in the databases compared with known photorespiratory genes (e.g. AGT1 and hydroxypyruvate reductase). These ESTs were present in some mixed tissue libraries, but, interestingly, all three Arabidopsis AGT2 genes appear to be expressed in developing seed tissues. ClustalW analysis indicates that all of the AGT2-like sequences are more closely related to one another than any representative is to AGT1 or to AlaAT sequences (Fig. 1A).

Although mammalian AGT2s are localized exclusively to mitochondria, each Arabidopsis AGT2 homolog contains a PTS1, suggesting peroxisomal localization (Fig. 1B). TargetP analysis predicts that each Arabidopsis AGT2 homolog is mitochondrial, although this program does not specifically recognize peroxisome-targeting signals (Emanuelsson et al., 2000).

To determine whether an Arabidopsis AGT2 homolog catalyzes GGT or AGT transaminations, the coding sequence of At4g39660 was subcloned into the bacterial expression vector pET-28. Recombinant At4g39660 was expressed in the soluble fraction at relatively high levels in *Escherichia coli*. Lysates of bacteria expressing this construct contained no additional GGT, AGT, SGT, AKT, or GPT activity, compared with empty vector controls (data not shown). The failure to detect any aminotransferase activity associated with the expression of this cDNA could indicate that this protein does not catalyze any of the reactions assayed, or that the protein was improperly folded in *E. coli*, resulting in its inactivity. Gross misfolding of the enzyme is probably not the cause of the observed lack of activity because the recombinant protein was soluble and not in inclusion bodies.

Purification and Identification of Arabidopsis GGT

Because the reverse genetics approach was unsuccessful in identifying an enzyme catalyzing GGT/AGT reactions, GGT activity was chromatographically purified from Arabidopsis leaf tissue. A gel depicting the progress of the protein purification is shown in Figure 2; the final purification factor was greater than 50-fold (Table I).

A protein of approximately 55 kD copurified with the GGT activity peak at each step of the purification. After hydroxylapatite chromatography, this protein was excised from a Coomassie Blue-stained gel and subjected to trypsin digestion and matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) analysis. The spectrum obtained for the tryptic fragments of the purified protein was used to query the nr database, resulting in the identification of a pair of putative AlaATs from Arabidopsis with high degrees of peptide coverage. GGT1, the most likely candidate, containing peptides corresponding to 19 of 19 predicted peaks (covering 41% of the predicted protein sequence; Table II; Fig. 3A), matched AGI locus At1g23310. Another candidate, GGT2, containing peptides corresponding to 16 of 19 peaks (covering 37% of the predicted protein sequence), corresponded to AGI locus At1g70580. No other proteins contained peptides corresponding to more than four peaks, including both of the other two predicted AlaAT homologs in the Arabidopsis genome, AGI loci At1g72330 and At1g17290.

Analysis of the GGT1 and GGT2 sequences revealed that these proteins are 93% identical and 99%

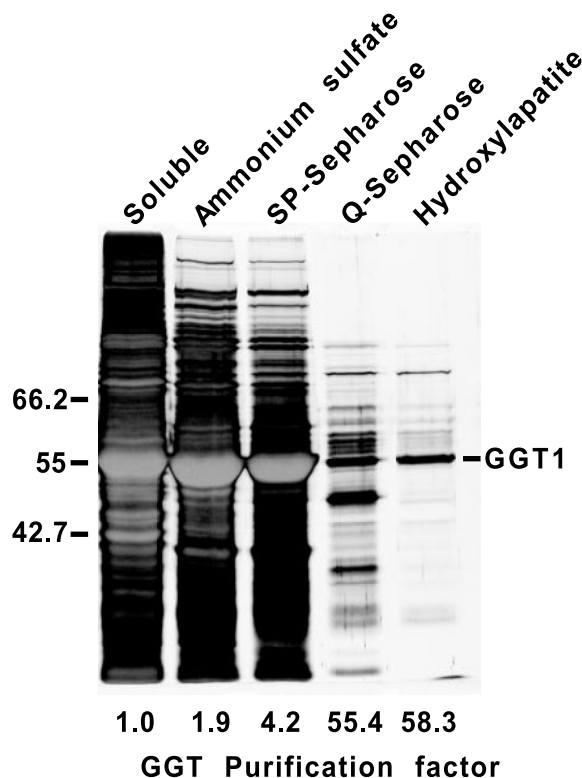


Figure 2. Purification of GGT activity from Arabidopsis leaves. GGT activity was chromatographically purified from Arabidopsis leaves after ammonium sulfate precipitation as described in "Materials and Methods." Samples containing approximately equal amounts of GGT activity were separated by SDS-PAGE and silver stained. The size of molecular mass standards (in kD) are indicated along the left side. The purification factor relative to the initial soluble fraction is indicated below the corresponding lane of the gel. Note that the large subunit of Rubisco obscures the GGT1 band before the Q-Sepharose purification step.

similar at the amino acid level. GGT1 and GGT2 are also closely related to the two other Arabidopsis AlaAT isozymes and to human AlaAT (Figs. 1A and 3B). The high degree of sequence conservation between GGT1 and GGT2 is probably because of a segmental duplication of the region of Arabidopsis chromosome I containing these two genes (AGI, 2000). Each of the predicted proteins is 481 amino acids in length, contains a protein family class I aminotransferase signature, and a conserved variant of the PTS1 (Olsen, 1998), suggesting peroxisomal localization. The other two putative Arabidopsis AlaATs (also segmentally duplicated) are predicted to be mitochondrial (AtAlaATm, AGI no. At1g72330) and cytosolic (AtAlaATc, AGI no. At1g17290) isozymes. For each of the putative organellar isozymes, the targeting information is appended as carboxyl- or amino-terminal extensions. The PTS1 sequence of the two putative peroxisomal isozymes resides on short carboxyl-terminal extensions, not present on the other isozymes, whereas the putative mitochondrial isozyme contains a long amino-terminal extension

Table I. Purification of GGT activity from Arabidopsis

Sample	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	units	units mg ⁻¹	-fold	%
Leaf extract	133.7	13.1	0.10	1.0	100
45% to 60% (w/v) (NH ₄) ₂ SO ₄	44.3	8.1	0.18	1.9	62
SP-Sepharose	16.5	6.7	0.41	4.2	51
Q-Sepharose	0.52	2.8	5.43	55.4	21
Hydroxylapatite	0.14	0.8	5.71	58.3	6

(Fig. 3A). Based on sequence alignment and comparison with solved structures containing the aminotransferase class I signature, residues G224, D249, K291, and R444 of GGT1 and GGT2 correspond to the conserved residues involved in PLP cofactor binding and enzymatic catalysis.

Expression of Arabidopsis GGT1 and GGT2

The sequences of GGT1 and GGT2 were both used to search for corresponding Arabidopsis ESTs. Over 70 ESTs, including several potential full-length clones corresponding to GGT1, were identified. These GGT1 ESTs were present in Arabidopsis cDNA libraries produced from mixed tissues, aboveground organs, green siliques, roots, flower buds, and leaves of salt-treated plants (Newman et al., 1994; Asamizu et al., 2000). Only two potential EST sequences corresponding to GGT2 were discovered, including a single potential full-length cDNA. Many additional cDNAs encoding AlaAT homologs from other organisms including *Chlamydomonas reinhardtii*, rice, *P. miliaceum*, barley, corn, human, rat, trypanosome (*Trypanosoma brucei*), yeast (*Saccharomyces cerevisiae*),

Caenorhabditis elegans, and *Drosophila melanogaster* are also present in the databases. Reverse transcriptase (RT)-PCR analysis with gene-specific primers demonstrated that GGT1 and GGT2 transcripts are detected in Arabidopsis seedlings grown in the light or in darkness (Fig. 4). GGT1 and GGT2 transcripts were also detected in mature leaves and green siliques of Arabidopsis; however, only GGT2 transcripts were detected in roots, where GGT1 transcripts were not observed. Although GGT1 transcripts were observed in 2- and 4-d-old plate-grown seedlings grown in continuous light or darkness, GGT2 transcripts were not detected in 4-d-old dark-grown seedlings. The transcript expression patterns observed for GGT1 and GGT2 were similar to that of AGT1 (Fig. 4), a photorespiratory gene (Liepman and Olsen, 2001).

Enzymatic Activity of Arabidopsis GGT1 and GGT2

The EST 70G6T7 was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus) because it contained the entire predicted coding sequence of GGT1, including 5'- and 3'-untranslated regions (UTRs). The 1,714-bp cDNA contained a 1,443-bp coding sequence that is flanked by 147 nucleotides of 5'-UTR and 124 nucleotides of 3'-UTR, and is predicted to encode a polypeptide of 53.3 kD. An in-frame stop codon, located one codon upstream of the predicted start codon, suggests that this cDNA contains the complete coding sequence of GGT1. To determine the activity of the enzyme encoded by this cDNA, the coding sequence of GGT1 was subcloned into the bacterial expression vector pET-28. Recombinant GGT1 was soluble when expressed in *E. coli*. Lysates of bacteria expressing this construct contained significantly increased levels of GGT, GPT, and AKT activity compared with empty vector controls (Fig. 5). AGT activity was also detectable, but GGT1 did not catalyze SGT or AspAT reactions (data not shown).

RT-PCR primers to amplify the coding sequence of GGT2 were designed using the sequence of the putative full-length cDNA (accession no. AY035130). The RT-PCR product was sequenced and corresponded perfectly to bases 107 to 1,688 of the putative full-length GGT2 cDNA. The 1,581-bp RT-PCR product contained 1443 bp of coding sequence flanked by 19 nucleotides of 5'-UTR and 119 nucleotides of 3'-UTR, and was predicted to encode a

Table II. MALDI-TOF analysis of purified Arabidopsis GGT

Peaks Observed	GGT1 Predicted Peaks
m/z	
750.44	750.4150
760.45	760.4317
787.47	787.4678
857.48	857.4634
969.60^a	969.5733
991.54	991.5213
1,055.43	1,055.4369
1,083.58	1,083.5549
1,119.63	1,119.6162
1,245.63	1,245.6812
1,381.65	1,381.6574
1,394.73	1,394.6803
1,397.66	1,397.6524
1,761.94	1,761.9387
1,783.81	1,783.8615
1,924.92	1,924.9180
1,982.96	1,982.9936
2,320.19	2,320.2930
2,854.37	2,854.4627

^aObserved peaks in bold indicate diagnostic peaks predicted for GGT1 but not for GGT2.

A.

AtGGT1	-----MAL	KALDYDTLNE	NVKKCQYAVR	GELYLRASEL	33
AtGGT2	-----MSL	KALDYESLNE	NVKNCQYAVR	GELYLRASEL	33
AtAlaATc	---MFCLVFFI	LTWFLFGGS	QVIKCEYAVR	GEIVNTAQKL	38
AtAlaATm	MRRFLINQAK	GLVDHSRRQH	HHKSPSFLSP	QPRPLASSPP	90
HsAlaAT	-----	-----	-----	-----	48
cons.	-----	-----	-----	-----	90
AtGGT1	QKEGKK----	-----IIFTN	VGNPHALGQK	PLTFPRQVVA	113
AtGGT2	QKEGKK----	-----IIFTN	VGNPHALGQK	PLTFPRQVVS	113
AtAlaATc	QEDLKTNKDA	YFPDEIIYCN	IGNPQSLGQQ	PIKPFREVL	128
AtAlaATm	QEDLKTNKDA	YFPDEIIYCN	IGNPQSLGQL	PIKPFREVL	180
HsAlaAT	EQELRQGVK	KPFTEVIRAN	IGDAQAMQQR	PIKPFRLQVLA	133
cons.***	180
AtGGT1	KEVAEPIQRR	DG-YPSDPEL	IFLTDGASKG	VMQILNCVIR	199
AtGGT2	KEVAEPIERR	DG-YPSDPEL	IFLTDGASKG	VMQILNCVIR	199
AtAlaATc	DAIADGIEAR	DG-FPADPND	IFMTDGASPG	VHMMQCLLIT	214
AtAlaATm	DVIAAGIEAR	DG-FPADPND	IFLTDGASPA	VHMMQCLLIS	266
HsAlaAT	EDVARYIERR	DGGIPADPNN	VFLTDGASDA	IVTVLKLVA	223
cons.****	270
AtGGT1	SVAQARSQGI	TVRAMVIINP	GNPTGQCLSE	ANIREILKFC	289
AtGGT2	SVAQARSQGI	TVRAMVIINP	GNPTGQCLSE	ANIREILKFC	289
AtAlaATc	QLEDARSKGI	TVRALAVINP	GNPTGQVLSL	ENQRDVVKFC	303
AtAlaATm	QLEEARSKGI	SVRALVINP	GNPTGQVLA	ENQRDVNFC	355
HsAlaAT	ALGOAR-DHC	RPRALCVINP	GNPTGQVQTR	ECIEAIVRFA	312
cons.****	360
AtGGT1	SKGYWGECGQ	RGGYFEMTNL	PPRVVEEIIYK	VASIALSPNV	379
AtGGT2	SKGYWGECGQ	RGGYFEMTNL	PPRVVEEIIYK	VASIALSPNV	379
AtAlaATc	SKGYWGECGK	RGGYMEVTGF	TSVDRECIYK	MASVNLCSNI	393
AtAlaATm	SKGYWGECGK	RGGYMEVTGF	TSVDRECIYK	MASVNLCSNI	445
HsAlaAT	SKGYWGECGF	RGGYVEVVM	DAVQQQMLK	LMSVRLCPPV	402
cons.****	450
AtGGT1	VVCNFTGEGAM	YSFPQIRLPT	GALQAQKQAG	KVPDVFYCLK	469
AtGGT2	VVCNFTGEGAM	YSFPQIRLPT	KATQAQKQAG	KVPDVFYCLK	469
AtAlaATc	VTCNRAEGAM	YLFPCILHLP	KATQAQKQAG	KVPDVFYCLK	483
AtAlaATm	VTCNRAEGAM	YLFPCILHLP	KATQAQKQAG	KVPDVFYCLK	535
HsAlaAT	ISCNPFVQAM	YSFPQVQLPP	RAVERAQELG	LAPDMFQCR	492
cons.****	540
AtGGT1	TQYDNNFGYS	KM	481		
AtGGT2	SQYADNFGYS	RM	481		
AtAlaATc	DEFRD----		488		
AtAlaATm	DEFRN----		540		
HsAlaAT	LEYS-----		496		
cons.	..		552		

B.

	AtGGT1	AtGGT2	AtAlaATc	AtAlaATm	HsAlaAT	
AtGGT1		99	78	71	74	% Similarity
AtGGT2	93		79	72	73	
AtAlaATc	49	48		87	73	
AtAlaATm	44	43	78		71	
HsAlaAT	43	42	45	43		
	% Identity					

Figure 3. Sequence analysis of Arabidopsis alanine aminotransferases. A, Two peroxisomal Arabidopsis AlaAT homologs (AtGGT1, AGI locus no. At1g23310; and AtGGT2, AGI locus no. At1g70580) were aligned with the putative cytosolic Arabidopsis AlaAT isozyme (AtAlaATc, AGI locus no. At1g17290), the putative mitochondrial Arabidopsis isozyme (AtAlaATm, AGI locus no. At1g72330), and human AlaAT (HsAlaAT, accession no. P24298) to produce a consensus sequence (cons.) using the ClustalW algorithm. Asterisks denote residues identical in all five sequences; dots indicate residues similar in at least three of the sequences. Black circles indicate the four residues that best conform to the aminotransferase signature; K291 is the predicted PLP cofactor-binding site of GGT1. A solid bar above the carboxyl terminus of GGT1 and GGT2 indicates the putative PTS1. Residues in bold in the GGT1 sequence correspond to tryptic fragments identified with MALDI-TOF analysis of purified protein with GGT activity from the hydroxylapatite fraction. B, Pair-wise sequence similarity and identity between Arabidopsis and human AlaAT homologs. Percent similarity between two sequences is shown above the diagonal line; percent identity is shown below the diagonal line.

protein of 53.4 kD. An in-frame stop codon, located four codons upstream of the predicted start codon, indicates that this cDNA contains the complete cod-

ing sequence of GGT2. Recombinant GGT2 was soluble when expressed in *E. coli*; lysates of bacteria expressing this construct exhibited significantly in-

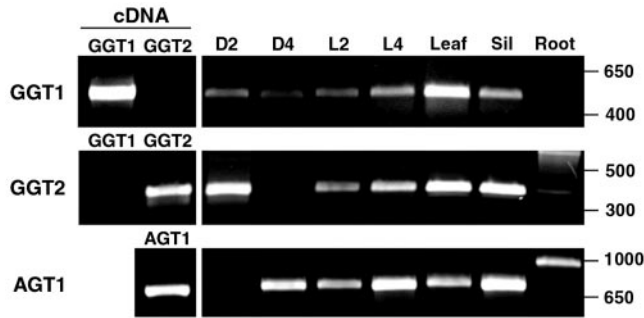


Figure 4. Gene-specific RT-PCR analysis of AtGGTs. RT-PCR with gene-specific primers was used to examine AtGGT transcript presence in total RNA samples prepared from 2- and 4-d-old dark-grown (D2 and D4) and light-grown (L2 and L4) Arabidopsis seedlings, as well as mature leaves, green siliques (sil), and roots. RT-PCR products were separated on 1.5% (w/v) agarose gels and visualized with ethidium bromide in UV light. The cDNA lanes in the left represent PCR reaction products using the indicated cDNAs as the template and the same gene-specific primers as were used in the corresponding RT-PCR reactions. The positions of molecular mass standards (in bp) are indicated to the right of each gel. No RT-PCR product corresponding in size to the cDNA control was observed in control reactions lacking RT; the larger band seen with AGT1 primers and root mRNA is probably because of amplification of the genomic sequence in the absence of transcript. All reactions were repeated at least twice, with similar results.

creased levels of GGT, GPT, and AKT activity compared with empty vector controls (Fig. 5).

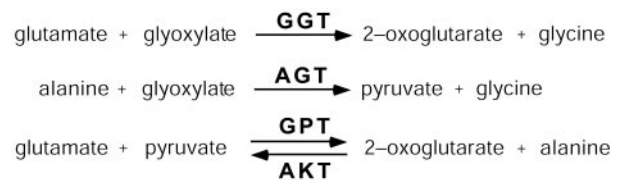
To compare the enzymatic activity of these two nearly identical aminotransferases, recombinant GGT1 and GGT2 were chromatographically purified to near homogeneity (Fig. 5). Both purified recombinant proteins catalyzed GGT, GPT, and AKT reactions (Fig. 5B; Table III). These purified aminotransferases had very similar K_m values for the amino acid substrates Glu and Ala, as well as the 2-oxoacid substrates glyoxylate, pyruvate, and 2-oxoglutarate. The purified recombinant aminotransferases also had similar kinetic characteristics compared with the protein purified from Arabidopsis leaves (Table III). Because aminotransferase reactions proceed via a bimolecular ping-pong mechanism (Velick and Vavra, 1962; Henson and Cleland, 1964; Nakamura and Tolbert, 1983), where the amino acid and 2-oxoacid substrates bind sequentially, GGT1 and GGT2 were also expected to exhibit AGT activity. Both recombinant proteins and the purified plant protein displayed AGT activity (data not shown), but this activity was not characterized kinetically because of technical limitations of the AGT assay. Neither of the purified recombinant GGTS nor the purified Arabidopsis protein exhibited SGT or AspAT activity (data not shown).

Subcellular Localization of GGT Activity

Plant AlaATs are localized in multiple cellular locations including the cytosol, peroxisomes, and mi-

tochondria. GGT activity has been found in peroxisomes and the cytosol of various plant species, and also within algal mitochondria. To determine the subcellular localization of GGT activity in Arabidopsis, extracts of young seedlings were subjected to Suc gradient fractionation. Fractions were assayed for organellar marker enzyme activities, including catalase as a peroxisomal marker, fumarase as a mitochondrial marker, and chlorophyll to indicate chloroplasts. Fractions were also assayed for GGT activity. Peak GGT activity cofractionated with the catalase peak, very little GGT activity cofractionated with the mitochondrial and chloroplast peaks (Fig. 6). The GGT activity observed in Arabidopsis peroxisomes is likely the sum of the contributions from

A.



B.

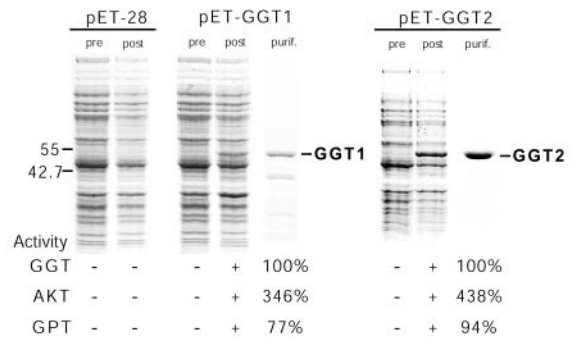


Figure 5. Expression, purification, and enzymatic characterization of AtGGT1 and AtGGT2. A, Four aminotransferase reactions catalyzed by GGT1 and GGT2: GGT, GPT, AKT, and AGT. Transaminations involving glyoxylate are thought to be irreversible because of low affinity of glyoxylate aminotransferases for Gly (Nakamura and Tolbert, 1983; Leegood et al., 1995) or thermodynamic reasons (Smith, 1985). B, Lysates of bacteria (approximately 100 μ g of protein) expressing the empty pET-28 vector, or this pET vector containing the AtGGT1 or AtGGT2 cDNA, were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Total cellular protein was collected before induction with isopropyl- β -D-thiogalactopyranoside (IPTG; pre), or 4 h after induction with IPTG (post). Recombinant AtGGT1 and AtGGT2 were chromatographically purified as described in "Materials and Methods"; approximately 11 μ g of purified recombinant GGT1 and 18 μ g of purified GGT2 are shown. The presence (+) or absence (-) of various aminotransferase activities in the whole-cell lysates or purified protein preparations is indicated below the gels. The activity of purified recombinant AtGGT1 and AtGGT2 for each aminotransferase reaction relative to the GGT reaction is shown below each purified protein. Because the recombinant proteins were at different concentrations, a specific activity of 15.5 units GGT activity mg protein^{-1} was set as 100% relative activity for AtGGT1, whereas a specific activity of 26.9 units GGT activity mg protein^{-1} was set as 100% relative activity for AtGGT2.

Table III. Kinetic parameters of purified *Arabidopsis* GGTs

Reaction	Amino Acid K_m	2-Oxoacid Concentration	2-Oxoacid K_m	Amino Acid Concentration
<i>mM</i>				
<i>Arabidopsis</i> GGT ^a				
GGT	3.00	5	0.27	10
GPT	—	—	0.33	10
AKT	7.16	2.5	0.27	20
Recombinant GGT1 ^b				
GGT	1.97	5	0.21	10
GPT	—	—	0.31	10
AKT	4.76	2.5	0.31	20
Recombinant GGT2 ^c				
GGT	3.32	5	0.14	10
GPT	—	—	0.36	10
AKT	3.56	2.5	0.51	20

^aPurified from *Arabidopsis* leaves. ^bPurified from lysate of *E. coli* expressing pET-GGT1. ^cPurified from lysate of *E. coli* expressing pET-GGT2.

GGT1 and GGT2 because both catalyze this reaction. It was not possible to determine the individual contribution of each enzyme to the total activity in peroxisomes using activity assays; however, GGT1 probably catalyzes most of the activity based on the comparative EST abundance of these genes. These subcellular fractionation data are consistent with a solely peroxisomal localization of GGT activity in *Arabidopsis*. The lack of GGT activity outside of the peroxisomes also suggests that the two other AlaAT isozymes of *Arabidopsis* do not catalyze this transamination, but may be specific for the GPT/AKT reaction.

DISCUSSION

Two peroxisomal glyoxylate aminotransferases are central to the photorespiratory pathway; one catalyzes the SGT transamination, the other catalyzes GGT and AGT transaminations (Fig. 7). Until recently, the identity of the corresponding enzymes was unknown. We previously demonstrated that a peroxisomal AGT1 homolog catalyzes the SGT reaction in *Arabidopsis* (Liepman and Olsen, 2001). The isolation and characterization of two *Arabidopsis* AlaATs with glyoxylate aminotransferase activity (GGT1 and GGT2) are presented here.

The amino acid sequences of GGT1 and GGT2 are 99% similar, probably because of a segmental duplication of the region of chromosome I containing these genes (AGI, 2000). GGT1 and GGT2 are also closely related to putative mitochondrial and cytosolic AlaAT isozymes in *Arabidopsis*. GGT1 and GGT2 both catalyze at least four aminotransferase reactions (Fig. 5), the two glyoxylate transaminations GGT and AGT, and the reversible AKT/GPT transamination that is characteristic of AlaATs. Kinetic analyses of GGT1 and GGT2 indicate that the amino acid substrates Glu and Ala, and the 2-oxoacids glyoxylate, 2-oxoglutarate, and pyruvate, are all possible in vivo

substrates of these enzymes (Table III). Where the concentrations of these metabolites have been measured in plant cells, the K_m values of GGT1 and GGT2 for these substrates fall well within the observed ranges (Winter et al., 1993, 1994). The similar transcript expression patterns (Fig. 4) and enzymatic activities of GGT1 and GGT2 indicate that these enzymes may serve partially redundant functions in *Arabidopsis*.

GGT activity was localized exclusively to *Arabidopsis* peroxisomes (Fig. 6). The discovery of two peroxisomal AlaAT isozymes in *Arabidopsis* is consistent with observations with spinach (*Spinacia oleracea*) leaves; however, in spinach, one form catalyzed the GGT reaction and the other did not (Noguchi and Hayashi, 1981).

Animals contain two forms of glyoxylate aminotransferases: AGT1 and mitochondrial AGT2. Because an AGT1 homolog catalyzes the SGT reaction of photorespiration in *Arabidopsis* (Liepman and Olsen, 2001), a logical candidate for another peroxisomal glyoxylate aminotransferase was an AGT2 homolog. The *Arabidopsis* genome contains three AGT2 homologs, each with a putative PTS1 (Fig. 1). The discovery of three potentially peroxisomal AGT2 homologs in plants is novel because animals contain a single AGT2 that catalyzes aminotransferase reactions with a wide range of substrates (Noguchi et al., 1978; Takada and Noguchi, 1980; Noguchi and Mori, 1981; Okuno et al., 1982; Ogawa et al., 1990; Kontani et al., 1993). The localization of AGT2 appears to be limited to mitochondria in animals, where its physiological role remains unclear (Takada and Noguchi, 1980; Noguchi and Mori, 1981; Ogawa et al., 1990; Lee et al., 1995).

When expressed in *E. coli*, one of the *Arabidopsis* AGT2 homologs did not appear to catalyze any of the glyoxylate aminotransferase or AlaAT activities assayed. It was not possible to determine whether this lack of activity resulted from enzymatic inactivity or

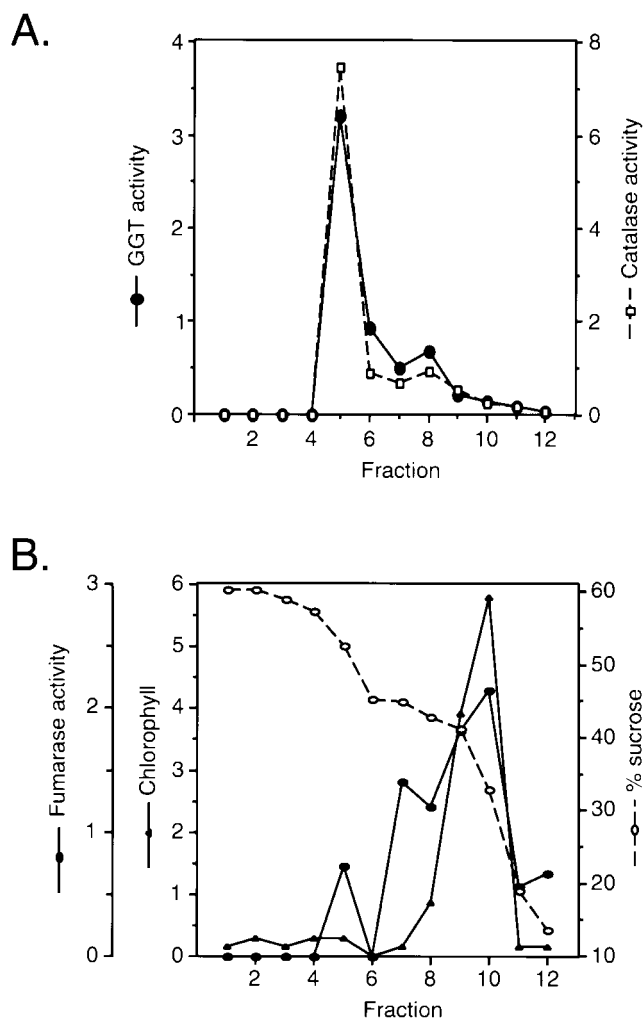


Figure 6. Localization of GGT activity to Arabidopsis peroxisomes by subcellular fractionation. Extracts of 2-week-old light-grown Arabidopsis seedlings were separated by Suc density centrifugation. A, Each fraction was assayed for GGT activity and the peroxisomal marker enzyme catalase as described in "Materials and Methods." B, Gradient fractions were also assayed for the mitochondrial marker fumarase, chloroplast marker chlorophyll, and Suc content. The specific activity (in $\mu\text{mol min}^{-1} \text{mg}^{-1}$ total protein) of each enzyme is shown (catalase $\times 10^4$, fumarase $\times 10^8$, and GGT $\times 10^7$); chlorophyll is expressed as mg chlorophyll mg^{-1} protein. Fraction 1 is from the bottom of the gradient; fraction 12 is the top.

if the recombinant enzyme was active but does not catalyze the assayed reactions. Plants containing mutations in one or more of these genes may provide another means to examine the physiological significance of these enzymes. If one or more of these *Arabidopsis* AGT2 homologs is peroxisomal, as suggested by the PTS1 in each sequence (Fig. 1), it might indicate that the range of aminotransferase reactions taking place within peroxisomes is more diverse than previously thought (Fig. 7). Another possibility is that one or more of the *Arabidopsis* AGT2 homologs are actually mitochondrial, which would be consistent with the observation of AGT activity in bean

(*Phaseolus vulgaris*) mitochondria (Roberts and Lees, 1997). In this case, the presence of a conserved variant of the PTS1 in all three *Arabidopsis* AGT2 homologs is even more curious.

Somerville and his colleagues carried out extensive mutagenesis studies on *Arabidopsis*, in search of mutations in proteins of the photorespiratory pathway (Somerville and Ogren, 1982; Somerville, 2001). Although mutations were recovered for many steps of the pathway, including another peroxisomal glyoxylate aminotransferase (Somerville and Ogren, 1980; Liepman and Olsen, 2001), plants lacking GGT or AGT activity were never reported. Because *Arabidopsis* contains two genes encoding very similar GGTs, it is highly unlikely that such double mutant plants could be isolated with their screening method. GGT1 and GGT2 transcripts are present in etiolated and light-grown *Arabidopsis* seedlings (Fig. 4), and GGT activity has been observed in etiolated cucumbers (*Cucumis sativus*), where it increases in response to greening (Noguchi and Fujiwara, 1982). These observations suggest that these proteins may be active in additional pathways other than photorespiration; thus, a mutation in one or both of these genes could prove lethal. It will be interesting to investigate the phenotypes of plants with disruptions within the GGT1 and GGT2 genes. Using single and double mutant plants, it should be possible to define the

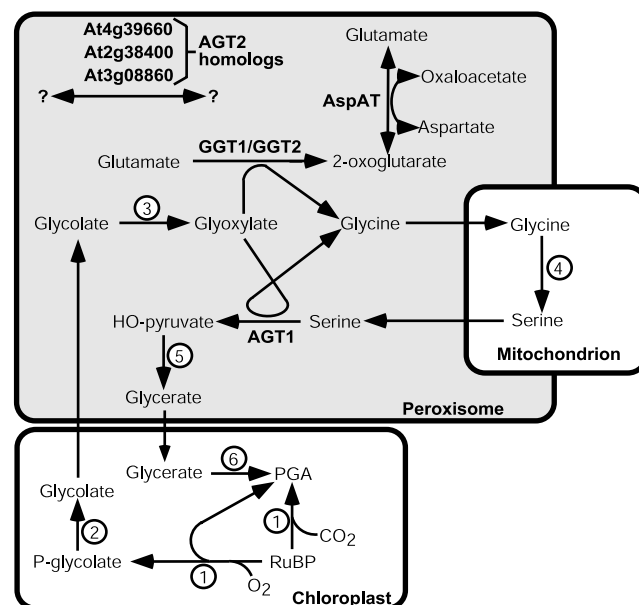


Figure 7. The photorespiratory peroxisomal glyoxylate aminotransferases, and other possible aminotransferase reactions in plant peroxisomes. Photorespiration involves reactions and metabolite exchange between chloroplasts, peroxisomes, and mitochondria. Key aminotransferases catalyzing some of these reactions in peroxisomes are indicated. Numbered enzyme key: 1, Rubisco; 2, phosphoglycolate phosphatase; 3, glycolate oxidase; 4, Gly decarboxylase/Ser transhydroxymethyltransferase; 5, hydroxypyruvate reductase; and 6, glycerate kinase. PGA, 3-Phosphoglycerate; P-glycolate, 2-phosphoglycolate; RuBP, ribulose 1,5-bisphosphate.

precise roles of each GGT. If double mutants have no GGT activity, this would rule out the possibility that one or more of the Arabidopsis AGT2 homologs also catalyzes this reaction.

Because of their relatively high degree of sequence conservation, Arabidopsis AlaATs may be good targets for directed evolution experiments to alter substrate specificity. Several groups have successfully changed the substrate specificity of bacterial AspAT (Yano et al., 1998; Graber et al., 1999; Oue et al., 1999). Many AlaATs, including presumably the mitochondrial and cytosolic isozymes of Arabidopsis, are specific for the reversible AKT/GPT transamination, and do not accept glyoxylate as an amino acceptor (Rech and Crouzet, 1974; Gazeu-Reyjal and Crouzet, 1976; Good and Muench, 1992). In addition to AKT and GPT transaminations, Arabidopsis GGT1 and GGT2 also catalyze GGT and AGT reactions. Although these aminotransferases are closely related to the other AlaATs in Arabidopsis, there are several regions where the sequences diverge. Thus, it may be possible to alter the substrate specificity of Arabidopsis AlaATs to add new 2-oxoacid substrates to the range of these enzymes, without significantly changing the overall affinity of these proteins for their native substrates.

The two commonly described glyoxylate transaminations of the photorespiratory pathway are the SGT and GGT reactions (Fig. 7). Arabidopsis GGT1 and GGT2 both catalyze an AGT reaction in addition to the GGT reaction, consistent with the work of a number of groups (Noguchi and Hayashi, 1981; Nakamura and Tolbert, 1983; Yu et al., 1984), but the physiological significance of the AGT reaction for photorespiration has been disputed (Betsche, 1983; Yu et al., 1984). Estimates based upon whole-leaf labeling analysis suggested that Ala contributed 3 times more amino groups to photorespiratory Gly formation than Glu did (Betsche, 1983), whereas another study involving feeding metabolites to purified peroxisomes concluded that Glu contributed more to glyoxylate transamination than did Ala (Yu et al., 1984). It is noteworthy that both Glu and Ala concentrations in the cytosol of leaves exposed to light are very high (3–90 mM; Winter et al., 1993, 1994), and that the K_m values of GGT1 and GGT2 for these substrates fall near the lower limit of this range (Table III). The concentrations of Glu and Ala in the cytosol, however, may not reflect the concentrations of these metabolites inside peroxisomes because the peroxisomal membrane is not freely permeable to these amino acids (Liang and Huang, 1983). Thus, the relative glyoxylate aminotransferase activity in planta with Glu and Ala may depend upon other factors determining the supply of these substrates, including metabolite transport and the activity of other anabolic and catabolic pathways.

Although the photorespiratory pathway has been the subject of many detailed investigations and the

proposed target for metabolic engineering strategies to increase crop productivity, genes encoding some of the enzymes within the pathway have only recently been identified. Future studies to more thoroughly define the physiological roles of these enzymes should benefit from the recent generation of plants with disruptions in these genes. Such studies, along with additional efforts to identify and characterize genes encoding the remaining unidentified photorespiratory enzymes, are necessary for a complete understanding of this physiologically significant pathway.

MATERIALS AND METHODS

Purification of Arabidopsis GGT

All purification steps were carried out at 4°C, unless otherwise noted. Five grams of Arabidopsis leaf tissue was pulverized in liquid nitrogen into a mortar and pestle. Frozen leaf powder was homogenized in a ground-glass homogenizer containing 15 mL of extraction buffer (50 mM MES-NaOH [pH 6], 0.2 mM PLP, 10% [v/v] glycerol, and 0.1% [v/v] Triton X-100). The plant extract was clarified twice by centrifugation at 29,000g for 10 min. Finely powdered ammonium sulfate was added incrementally to the cleared extract to bring the solution to 45% (w/v) saturation. Precipitated proteins were pelleted by centrifugation for 10 min at 29,000g and discarded. The supernatant was brought to 60% (w/v) saturation with ammonium sulfate. Precipitated proteins were recovered by centrifugation for 10 min at 29,000g. The 45% to 60% (w/v) pellet was resuspended in 2.5 mL of extraction buffer, and desalted using a PD-10 column (Pharmacia Biotech, Piscataway, NJ) before SP-Sepharose FF (Pharmacia Biotech) chromatography. GGT displayed very low affinity for SP-Sepharose media, and was eluted with 50 mM MES-NaOH (pH 6), 10% (v/v) glycerol, and 0.2 mM PLP. Fractions containing GGT activity were pooled and concentrated using a Centricon YM-30 centrifugal concentrator device. The buffer was exchanged for 50 mM Tris-HCl (pH 8), 10% (v/v) glycerol, and 0.2 mM PLP (buffer A) using a PD-10 column. The sample was applied to a Q-Sepharose FF (Pharmacia Biotech) column and eluted with a linear ammonium sulfate gradient (0–150 mM) in buffer A. Fractions containing peak GGT activity were pooled, concentrated, and buffer exchanged for buffer B (50 mM Tris-HCl [pH 7.1], 10% [v/v] glycerol, and 10 mM potassium phosphate). The sample was applied to a hydroxylapatite (Bio-gel HTP, Bio-Rad Laboratories, Hercules, CA) column, and eluted with a linear gradient of potassium phosphate (10–200 mM) in buffer B.

MALDI-TOF Analysis

After the hydroxylapatite chromatography step, a Coomassie Blue-stained protein band of approximately 54 kD cofractionating with peak GGT activity throughout the purification procedure was excised from an acrylamide gel after SDS-PAGE. An in-gel trypsin digestion was performed using 500 ng of modified porcine trypsin (Promega, Madison, WI) as described (Phadke et al., 2001). Sample preparation and MALDI-TOF analysis followed the procedure of Phadke et al. (2001) with the following modification: Peptide mass fingerprints were searched against the Arabidopsis proteins of the National Center for Biotechnology Information database (4/19/01 version) using the MS-Fit program (Protein Prospector package; Clauser et al., 1999; <http://prospector.ucsf.edu/>), with a mass accuracy of 50 ppm. For the search, trypsin digestion with a maximum of two missed enzymatic cleavages, unmodified Cys, modification by acrylamide, peptide N-terminal Gln to pyro-Glu, Met oxidation, and protein N-terminal acetylation were considered.

Primer Sequences

The following sequences correspond to primers used for cloning procedures and RT-PCR. Sequences in bold indicate restriction sites designed to facilitate specific cloning steps described below: At4g39960-*NcoI*,

5'-CGAATCCATGGCGTTACAAAGGC-3'; GGT1-*NcoI*, 5'-CTGACCATGCGCTCTCAAGGCATTAGACTAC-3'; GGT1-*HindIII*, 5'-CTGAAAGCTTGTAATCACATTTTCGAATAACC-3'; GGT1-P6, 5'-TGGATACCCATCACGCGGTG-3'; GGT1-RT3, 5'-ATATTTTAACTCTCCCACTC-3'; GGT2-*NcoI*, 5'-CCCATGGCTCTCAAGGCCTTAGACTAC-3'; GGT2-*HindIII*, 5'-GAAGCTTAGTGATCTCTGACTCTGAC-3'; GGT2-P4, 5'-CTCAGCGACTTCTTCCGAAC-3'; GGT2-RT3, 5'-TCTACTTGCCTGCTCTGGAAC-3'; GGT2-RT4, 5'-GAGTTTGACACAGAGTAGGACCAG-3'; AGT1-P3, 5'-GCTTGCTCTGGATTGACCAGCAG-3'; and AGT1-P4, 5'-CCAGCCACTTTGTGAGACCAAG-3'.

RT-PCR Analysis

Arabidopsis seedlings were grown on germination plates (Olsen et al., 1993). Total RNA was collected 2 and 4 d after germination from whole etiolated seedlings and seedlings grown under continuous light. Total RNA was also collected from mature Arabidopsis leaves, siliques, and roots using the RNeasy plant mini-kit (Qiagen USA, Valencia, CA). RNA was analyzed on agarose:formaldehyde gels (Liepman and Olsen, 2001) and quantitated spectrophotometrically. RT-PCR analysis was conducted using the Access RT-PCR system and manufacturer's recommended conditions (Promega). One hundred nanograms of total RNA from the specified tissues was used as template. RT-PCR products corresponding in size to the cDNA products were absent in control samples lacking RT (data not shown). The RT-PCR primer combinations and predicted product sizes were: GGT1-RT3/GGT1-P6 to amplify a 522-bp *GGT1* fragment, GGT2-RT3/GGT2-P4 to amplify a 471-bp *GGT2* fragment, and AGT1-P3/AGT1-P4 to amplify a 743-bp *AGT1* fragment. RT-PCR products were separated on 1.5% (w/v) agarose gels, and stained with ethidium bromide. The RT-PCR results shown are from one experiment that was repeated at least two times with similar results.

Constructs

The EST 229N6T7 was selected and obtained from the Arabidopsis Biological Resource Center EST collection (Newman et al., 1994) because it appeared to contain the complete coding sequence of an Arabidopsis AGT2 homolog. The pZL-229N6T7 plasmid contains the cDNA of AGT At4g39960 in the pZL1 vector (Life Technologies/Gibco-BRL, Cleveland). PCR with *Taq* polymerase (Promega) using the At4g39960-*NcoI* primer and the SP6 primer was used to introduce a unique *NcoI* site for cloning purposes. The resulting PCR product was ligated with the pCR-II vector producing the plasmid pCR-At4g39960. An *NcoI/HindIII* fragment was subcloned from pCR-At4g39960 into the pET-28 vector (Novagen, Madison, WI), producing the plasmid pET-At4g39960.

The EST 70G6T7 was selected and obtained from the Arabidopsis Biological Resource Center EST collection because it appeared to contain the complete coding sequence of Arabidopsis GGT1. The plasmid pZL-70G6T7 contains the GGT1 cDNA in the pZL1 vector (Life Technologies/Gibco-BRL). PCR with *Taq* polymerase (New England Biolabs, Beverly, MA) using the GGT1-*NcoI*/GGT1-*HindIII* primer combination and the GGT1 cDNA was used to introduce restriction sites for cloning purposes. This PCR product was incubated with *Taq* polymerase (Promega) for 30 min at 72°C to add non-template deoxyadenosine overhangs to the 3' ends, and ligated with the pCR-II vector (Invitrogen, Carlsbad, CA), producing the plasmid pCRII-GGT1. The *NcoI/HindIII* fragment of pCRII-GGT1 was subcloned into the corresponding sites of the pET-28 vector (Novagen) to produce pET-GGT1.

To isolate GGT2 cDNA, RT-PCR was performed using the Access RT-PCR system (Promega) with the manufacturer's recommended conditions. The primer combination used was GGT2-RT3/GGT2-RT4, and the template was 100 ng of total RNA collected from Arabidopsis seedlings grown on germination plates (Olsen et al., 1993) for 9 d with continuous light. The RT-PCR product was excised from an agarose gel, purified using the GeneClean Spin Kit (Bio 101, Vista, CA), and ligated to the pCR-II vector (Invitrogen) following manufacturer's instructions. The plasmid pCRII-GGT2 contains the GGT2 cDNA in the pCR-II vector. PCR with *Taq* polymerase (New England Biolabs) using the GGT2-*NcoI*/GGT2-*HindIII* primer combination and the GGT2 cDNA was used to introduce restriction sites for cloning purposes. This PCR product was incubated with *Taq* polymerase (Promega) for 30 min at 72°C to add non-template deoxyadenosine overhangs to the 3'

ends, and ligated with the pCR-II vector to produce the plasmid pCRII-GGT2-*NcoI/HindIII*. The addition of an *NcoI* site containing the start codon results in the obligatory substitution of an Ala for a Ser at the second amino acid position of GGT2. The *NcoI/HindIII* fragment of pCRII-GGT2-*NcoI/HindIII* was subcloned into the corresponding sites of the pET-28 vector (Novagen) to produce pET-GGT2.

Both strands of cDNA clones of GGT1 and GGT2 from Arabidopsis were fully sequenced and the sequences deposited in GenBank: GGT1 (accession no. AF479639) and GGT2 (accession no. AF479640).

Recombinant GGT1 and GGT2 Expression and Purification from *Escherichia coli*

BL-21(DE3) cells were transformed with pET-GGT1, pET-GGT2, or the pET28 empty vector, and grown on Luria-Bertani (LB) plates supplemented with kanamycin. Single colonies were used to inoculate 3 mL of LB with kanamycin selection. One hundred microliters from the overnight culture was used to inoculate 250 mL of fresh LB media. Cells were shaken at 37°C until an OD₆₀₀ of approximately 0.5 was reached before the addition of 500 μ M IPTG to induce protein expression. After the addition of IPTG, cultures were supplemented with 50 μ M pyridoxine hydrochloride and shaken at 25°C. Four to 12 h after induction, cells were harvested by centrifugation at 4,068g at 4°C for 10 min. To test for protein expression and enzyme activity, bacterial whole-cell lysates were made from 1.5-mL aliquots of cells collected before and after induction with IPTG. Whole-cell lysates were prepared by resuspending the cells at a concentration of 5 OD₆₀₀ mL⁻¹ in cold lysis buffer (50 mM Tris-HCl [pH 7.1], 10 mM EDTA, 10 mM NaCl, 0.1 mM PLP, 10% [v/v] glycerol, 0.1% [v/v] β -mercaptoethanol, and 0.1% [v/v] Triton X-100). Cells were broken mechanically by repeated cycles of freezing and thawing in liquid nitrogen. GGT1 and GGT2 were purified from the soluble fraction of *E. coli* lysates prepared from 250-mL cultures as described above for the Arabidopsis leaf protein. Satisfactory purifications of GGT1 and GGT2 required only Q-Sepharose FF and hydroxylapatite chromatography.

Enzyme Assays

GGT, GPT, AGT, and AKT activities were measured spectrophotometrically in quartz cuvettes with a UV160U Spectrophotometer (Shimadzu, Columbia, MD) at 25°C. GGT activity was measured at 340 nm using an assay modified from Huang et al. (1976) by coupling the reductive amination of 2-oxoglutarate produced by GGT to the oxidation of NADH, catalyzed by excess bovine liver glutamic dehydrogenase (G2501, Sigma, St. Louis). To assay GGT activity, protein extract (5–10 μ L) was added to a 1-mL reaction mixture containing 60 mM potassium phosphate (pH 7.5), 5 mM glyoxylate, 0.17 mM NADH, and 2.2 units mL⁻¹ glutamic dehydrogenase. GPT activity was measured using conditions identical to the GGT assay except that 3 mM pyruvate replaced glyoxylate as the amino acceptor. AKT activity was measured at 340 nm by coupling the reduction of pyruvate to the oxidation of NADH, catalyzed by excess rabbit muscle lactate dehydrogenase (Sigma). To assay AKT activity, protein extract (5–10 μ L) was added to a 1-mL reaction mixture containing 70 mM HEPES-NaOH (pH 7), 2.5 mM 2-oxoglutarate, 0.17 mM NADH, and 5 units mL⁻¹ lactate dehydrogenase. AGT, SGT, and AspAT activities were assayed as described by Liepman and Olsen (2001) and Rehfield and Tolbert (1972). Although the AGT assay allowed for clear detection of AGT activity, increasing concentrations of glyoxylate inhibited the assay, limiting its utility for kinetic studies (Rehfield and Tolbert, 1972; Roberts and Lees, 1997). For each aminotransferase assay, the reaction was initiated by adding amino acid to a final concentration of 10 to 20 mM to the sample cuvette; water was added instead of amino acid in the reference cuvette. All assays were linear over the time course of the measurements. For kinetic measurements, substrate concentrations ranged from 0.5 to 100 mM for amino acids, and 0.025 to 5 mM for 2-oxoacids. Arabidopsis GGTs displayed typical Michaelis-Menten kinetics within these substrate ranges. K_m values were calculated from (at least) triplicate data points using nonlinear regression software (PRISM, GraphPad Software, San Diego). One unit of activity corresponds to the amount of enzyme converting 1 μ mol Glu to 2-oxoglutarate per minute under assay conditions.

Subcellular Fractionation

Subcellular fractionation was performed as previously described (Liepman and Olsen, 2001). GGT activity in Suc gradient fractions was assayed as described above except that 0.1% (v/v) Triton X-100 was included in the assay mixture to disrupt organellar membranes and ensure access to matrix proteins. Gradient fractions were analyzed for protein concentration, Suc content, and organellar marker enzymes as previously described (Liepman and Olsen, 2001).

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LITERATURE CITED

- AGI (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**: 796–815
- Asamizu E, Nakamura Y, Sato S, Tabata S (2000) A large scale analysis of cDNA in *Arabidopsis thaliana*: generation of 12,028 non-redundant expressed sequence tags from normalized and size-selected cDNA libraries. *DNA Res* **7**: 175–180
- Betsche T (1983) Amino transfer from alanine and glutamate to glycine and serine during photorespiration in oat leaves. *Plant Physiol* **71**: 961–965
- Biekmann S, Feierabend J (1982) Subcellular distribution, multiple forms and development of glutamate-pyruvate (glyoxylate) aminotransferase in plant tissues. *Biochim Biophys Acta* **721**: 268–279
- Buchanan BB, Gruissem W, Jones RL (2000) Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD
- Chang C-C, Huang AHC (1981) Metabolism of glycolate in isolated spinach leaf peroxisomes: kinetics of glyoxylate, oxalate, carbon dioxide, and glycine formation. *Plant Physiol* **67**: 1003–1006
- Chistoserdova L V, Lidstrom ME (1994) Genetics of the serine cycle in *Methylobacterium extorquens* AM1: identification of *sgaA*, and *mtidA* and sequences of *sgaA*, *hprA*, and *mtidA*. *J Bacteriol* **176**: 1957–1968
- Clauser KR, Baker PR, Burlingame AL (1999) Role of accurate mass measurement (+/-10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal Chem* **71**: 2871–2882
- Danpure C (1997) Variable peroxisomal and mitochondrial targeting of alanine: glyoxylate aminotransferase in mammalian evolution and disease. *Bioessays* **19**: 317–326
- Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* **300**: 1005–1016
- Gazeu-Reyjal M, Crouzet J (1976) Identite des formes soluble et solubilisee a partir des mitochondries de l'alanine α -cetoglutarate aminotransferase de *Lycopersicon esculentum*. *Phytochemistry* **15**: 1619–1622
- Good AG, Muench DG (1992) Purification and characterization of an anaerobically induced alanine aminotransferase from barley roots. *Plant Physiol* **99**: 1520–1525
- Graber R, Kasper P, Malashkevich VN, Strop P, Gehring H, Jansonius JN, Christen P (1999) Conversion of aspartate aminotransferase into an L-aspartate β -decarboxylase by a triple active-site mutation. *J Biol Chem* **274**: 31203–31208
- Hagashita T, Yoshida T, Izumi Y, Mitsunaga T (1996) Cloning and expression of the gene for serine:glyoxylate aminotransferase from an obligate methylotroph *Hyphomicrobium methylovorum* GM2. *Eur J Biochem* **241**: 1–5
- Henson CP, Cleland WW (1964) Kinetic studies of glutamic oxaloacetic transaminase isozymes. *Biochemistry* **3**: 338–345
- Hoffman NE, Bent AF, Hanson AD (1986) Induction of lactate dehydrogenase isozymes by oxygen deficit in barley root tissue. *Plant Physiol* **82**: 658–663
- Huang AHC, Liu KDF, Youle RJ (1976) Organelle-specific isozymes of aspartate- α -ketoglutarate transaminase in spinach. *Plant Physiol* **58**: 110–113
- Husic DW, Husic HD, Tolbert NE (1987) The oxidative photosynthetic carbon cycle or C₂ cycle. *Crit Rev Plant Sci* **5**: 45–100

- Kikuchi H, Hirose S, Toki S, Kazuhito A, Takaiwa F (1999) Molecular characterization of a gene for alanine aminotransferase from rice (*Oryza sativa*). *Plant Mol Biol* **39**: 149–159
- Kisaki T, Tolbert NE (1969) Glycolate and glyoxylate metabolism by isolated peroxisomes or chloroplasts. *Plant Physiol* **44**: 242–250
- Kontani Y, Kaneko M, Kikugawa M, Fujimoto S, Tamaki N (1993) Identity of D-3-aminoisobutyrate-pyruvate aminotransferase with alanine-glyoxylate aminotransferase 2. *Biochim Biophys Acta* **1156**: 161–166
- Lee IS, Muragaki Y, Ideguchi T, Hase T, Tsuji M, Ooshima A, Okuno E, Kido R (1995) Molecular cloning and sequencing of a cDNA encoding alanine-glyoxylate aminotransferase 2 from rat kidney. *J Biochem* **117**: 856–862
- Leegood RC, Lea PJ, Adcock MD, Hausler RE (1995) The regulation and control of photorespiration. *J Exp Bot* **46**: 1397–1414
- Liang Z, Huang AHC (1983) Metabolism of glycolate and glyoxylate in intact spinach leaf peroxisomes. *Plant Physiol* **73**: 147–152
- Liepman AH, Olsen LJ (2001) Peroxisomal alanine:glyoxylate aminotransferase (AGT1) is a photorespiratory enzyme with multiple substrates in *Arabidopsis thaliana*. *Plant J* **25**: 487–498
- Muench DG, Christopher ME, Good AG (1998) Cloning and expression of a hypoxic and nitrogen inducible maize alanine aminotransferase gene. *Physiol Plant* **103**: 503–512
- Muench DG, Good AG (1994) Hypoxically inducible barley alanine aminotransferase: cDNA cloning and expression analysis. *Plant Mol Biol* **24**: 417–427
- Nakamura Y, Tolbert NE (1983) Serine:glyoxylate, alanine:glyoxylate, and glutamate:glyoxylate aminotransferase reactions in peroxisomes from spinach leaves. *J Biol Chem* **258**: 7631–7638
- Newman T, de Bruijn FJ, Green P, Keegstra K, Kende H, McIntosh L, Ohlrogge J, Raikhel N, Somerville S, Thomashow M et al. (1994) Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous Arabidopsis cDNA clones. *Plant Physiol* **106**: 1241–1255
- Nishiyama K, Berstein G, Oda T, Ichijima A (1990) Cloning and nucleotide sequence of cDNA encoding human liver serine-pyruvate aminotransferase. *Eur J Biochem* **194**: 9–18
- Noguchi T, Fujiwara S (1982) Development of glutamate:glyoxylate aminotransferase in the cotyledons of cucumber (*Cucumis sativus*) seedlings. *Biochem J* **201**: 209–214
- Noguchi T, Hayashi S (1981) Plant leaf alanine:2-oxoglutarate aminotransferase. *Biochem J* **195**: 235–239
- Noguchi T, Mori R (1981) Biosynthesis of porphyrin precursors in mammals: identity of alanine: γ , δ -dioxovalerate aminotransferase with alanine:glyoxylate aminotransferase. *J Biol Chem* **256**: 10335–10339
- Noguchi T, Okuno E, Takada Y, Minatogawa Y, Okai K, Kido R (1978) Characteristics of hepatic alanine-glyoxylate aminotransferase in different mammalian species. *Biochem J* **169**: 113–122
- Ogawa T, Kimoto M, Sasaoka K (1990) Dimethylarginine:pyruvate aminotransferase in rats: purification, properties, and identity with alanine:glyoxylate aminotransferase 2. *J Biol Chem* **265**: 20938–20945
- Ogren WL (1984) Photorespiration: pathways, regulation, and modification. *Annu Rev Plant Physiol* **35**: 415–442
- Okuno E, Minatogawa Y, Kido R (1982) Co-purification of alanine-glyoxylate aminotransferase with 2-aminobutyrate aminotransferase in rat kidney. *Biochim Biophys Acta* **715**: 97–104
- Olsen LJ (1998) The surprising complexity of peroxisome biogenesis. *Plant Mol Biol* **38**: 163–189
- Olsen LJ, Ettinger WF, Damsz B, Matsudaira K, Webb MA, Harada JJ (1993) Targeting of glyoxysomal proteins to peroxisomes in leaves and roots of a higher plant. *Plant Cell* **5**: 941–952
- Orzechowski S, Socha-Hanc J, Paszkowski A (1999) Alanine aminotransferase and glycine aminotransferase from maize (*Zea mays* L.) leaves. *Acta Biochim Pol* **46**: 447–457
- Otter T, Penner JM, Mohr H (1992) Control of the appearance of alanine aminotransferase in the scots pine (*Pinus sylvestris* L.) seedling. *Planta* **188**: 376–383
- Oue S, Okamoto A, Yano T, Kagamiyama H (1999) Redesigning the substrate specificity of an enzyme by cumulative effects of the mutations of non-active site residues. *J Biol Chem* **274**: 2344–2349
- Paszkowski A, Niedzielska A (1989) Glutamate:glyoxylate aminotransferase from the seedlings of rye (*Secale cereale* L.). *Acta Biochim Pol* **36**: 17–29
- Penner JM (1991) Analysis of alanine and aspartate aminotransferase isoforms in mustard (*Sinapis alba* L.) cotyledons. *J Chromatogr* **587**: 101–108

- Phadke ND, Molloy MP, Steinhoff SA, Ulintz PJ, Andrews PC, Maddock JR** (2001) Analysis of the outer membrane proteome of *Caulobacter crescentus* by two-dimensional electrophoresis and mass spectrometry. *Proteomics* **1**: 705–720
- Rech J, Crouzet J** (1974) Partial purification and initial studies of the tomato L-alanine:2-oxoglutarate aminotransferase. *Biochim Biophys Acta* **350**: 392–399
- Rehfield DW, Tolbert NE** (1972) Aminotransferases in peroxisomes from spinach leaves. *J Biol Chem* **247**: 4803–4811
- Roberts TH, Lees EM** (1997) Glyoxylate aminotransferase activities in developing fruits of legumes. *J Biochem Mol Biol Biophys* **1**: 63–71
- Smith IK** (1985) Aminotransferases utilizing glyoxylate. In P Christen, DE Metzler, eds, *Transaminases*. John Wiley & Sons, New York, pp 390–396
- Somerville CR** (2001) An early Arabidopsis demonstration. Resolving a few issues concerning photorespiration. *Plant Physiol* **125**: 20–24
- Somerville CR, Ogren WL** (1980) Photorespiration mutants of *Arabidopsis thaliana* deficient in serine-glyoxylate aminotransferase activity. *Proc Natl Acad Sci USA* **77**: 2684–2687
- Somerville CR, Ogren WL** (1982) Genetic modification of photorespiration. *Trends Biochem Sci* **7**: 171–174
- Son D, Jo J, Sugiyama T** (1991) Purification and characterization of alanine aminotransferase from *Panicum miliaceum* leaves. *Arch Biochem Biophys* **289**: 262–266
- Son D, Sugiyama T** (1992) Molecular cloning of an alanine aminotransferase from NAD-malic enzyme type C4 plant *Panicum miliaceum*. *Plant Mol Biol* **20**: 705–713
- Takada Y, Noguchi T** (1980) Kidney alanine:glyoxylate aminotransferase isoenzymes; species distribution, subcellular distribution, and properties. *Comput Biochem Physiol B* **65B**: 133–138
- Velick SF, Vavra J** (1962) A kinetic and equilibrium analysis of the glutamic oxaloacetate transaminase mechanism. *J Biol Chem* **237**: 2109–2122
- Watson NR, Peschke VM, Russell DA, Sachs MM** (1992) Analysis of L-alanine:2-oxoglutarate aminotransferase isozymes in maize. *Biochem Genet* **30**: 371–383
- Winter H, Robinson DG, Heldt HW** (1993) Subcellular volumes and metabolite concentrations in barley leaves. *Planta* **191**: 180–190
- Winter H, Robinson DG, Heldt HW** (1994) Subcellular volumes and metabolite concentrations in spinach leaves. *Planta* **193**: 530–535
- Yano T, Oue S, Kagamiyama H** (1998) Directed evolution of an aspartate aminotransferase with new substrate specificities. *Proc Natl Acad Sci USA* **95**: 5511–5515
- Yokota A, Suehiro S, Kitaoka S** (1985) Purification and some properties of mitochondrial glutamate:glyoxylate aminotransferase and mechanism of its involvement in glycolate pathway in *Euglena gracilis* z. *Arch Biochem Biophys* **242**: 507–514
- Yu C, Liang Z, Huang AHC** (1984) Glyoxylate transamination in intact leaf peroxisomes. *Plant Physiol* **75**: 7–12